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Superantigen-induced CD4 Memory T Cell Anergy. I. Staphylococcal Enterotoxin B Induces Fyn-mediated Negative Signaling¹

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Abstract

Memory CD4 T cells must provide robust protection for an organism while still maintaining self-tolerance. Superantigens reveal a memory cell-specific regulatory pathway, by which signaling through the TCR can lead to clonal tolerance (anergy). Here we show that the src kinase Fyn is a critical regulator of anergy in murine memory CD4 T cells induced by the bacterial superantigen staphylococcal enterotoxin B (SEB). Exposure to SEB results in impaired TCR signaling due to failed CD3/ZAP-70 complex formation. Further, signal transduction through the TCR remains similarly blocked when anergic memory cells are subsequently exposed to agonist peptide antigen. Pharmacological inhibition or genetic elimination of Fyn kinase reverses memory cell anergy, resulting in SEB-induced cell proliferation. The mechanism underlying impaired TCR signaling and subsequent memory cell anergy must involve a Fyn signaling pathway given that the suppression of Fyn activity restores CD3/ZAP-70 complex formation and TCR proximal signaling.

Keywords

T lymphocytes; Immunological Memory; Superantigens; Anergy; Rodents; Signal Transduction

INTRODUCTION

Adaptive immunity is characterized by secondary immune responses that are mediated by B and T memory lymphocytes. Numerous differences distinguish memory cells from their precursor naive cells [1–4]. For CD4 T cells, these differences include alterations in phenotype and homing, and more vigorous function, as characterized by a increased range of secreted lymphokines. Consequently, memory responses to repeated antigen challenge are faster and more robust than are primary responses.

¹Abbreviations used: SEB, staphylococcal enterotoxin B; CFSE, 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester

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DISCLOSURES

The authors declare no conflict of interest or financial interests.

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Besides protecting the host from foreign pathogens, the immune system must recognize and not respond to self-antigens. This problem is especially acute for memory cells, which, by definition, are derived from naive cells that did not meet the threshold for removal by central tolerance mechanisms [5] and have instead successfully mounted a primary response to antigen. Memory T cells express higher levels of adhesion molecules and are generally more avid than are naive T cells [6–8]. Hence, cells with TCRs that cross-react with self-antigens could be more likely to interact with a host-derived antigen. Such cells, or self-reactive memory cells that arise either because of failed peripheral tolerance or non-specific activation of naive cells, can be particularly harmful to host tissues, due to their increased functional capacity. It is likely that additional or novel regulatory controls are imposed upon memory cells to prevent inappropriate actions.

Microbial superantigens stimulate vigorous proliferation by CD4 T cells expressing a given TCR V β [9–12]. In our previous studies, we showed that such activation was limited to naive CD4 T cells and antigen-stimulated effector cells, whereas resting memory cells were not stimulated by superantigens [13;14]. The selective failure of memory CD4 cells to respond to superantigens is reminiscent of other studies that showed that naive and memory cells could respond differently to the same stimulus. Most notably, several previous studies showed that soluble anti-CD3 preferentially stimulates naive cells, while blocking activation of memory cells [15;16]. In other studies, tolerance was specifically induced in memory cells exposed to low densities of cognate peptide antigens [17–19]. Finally, using both a TCR transgenic mouse model, as well as normal, non-transgenic mice, we further showed that exposure to superantigens, such as staphylococcal enterotoxin B (SEB), caused memory cells to become anergic and refractory to specific agonist peptide antigen [13;20]. Since soluble anti-CD3 did not lead to memory cell anergy, we have postulated that superantigens reveal a novel, memory cell-specific means of regulating cell activation.

In our subsequent studies, we examined signals transduced through the TCRs on memory cells in response to interactions with superantigens [21]. Previous studies had shown that superantigens, including SEB, could promote alternative signaling paths in either naive CD4 T cells or in cloned T cell lines [22–25]. In our studies on memory CD4 T cells, we found that SEB causes inappropriate proximal signaling through the TCR on memory cells, and that this effect results in a failure of the cells to proliferate or secrete lymphokines. Aberrant signaling in memory cells was characterized by a failure of the protein tyrosine kinase ZAP-70 to associate with TCR complex (pCD3 ζ) during antigen stimulation [21]. In the absence of such coupling, ZAP-70 was not phosphorylated by the src kinase Lck, and did not acquire kinase activity; thus, downstream signaling was blocked. That study also provided evidence that when SEB bound to the memory cell TCR, an alternative signaling pathway was involved. We observed slight increases in activation of a second src kinase Fyn, solely in memory cells that were exposed to SEB. The importance of elevated Fyn activity, if any, was unclear. In the present study, we have more fully examined the role of Fyn kinase in SEB-mediated anergy. We have found that Fyn is critical to this process, given that inhibition of Fyn activation rescued memory cells from anergy. Further, we have found that Fyn directly affects TCR proximal signaling in memory CD4 cells by regulating the association of ZAP-70 with the TCR complex and, thus, induces anergy.

MATERIALS AND METHODS

Animals

The BALB/c ByJ, DO11.10 [26], and DO11.10 x Fyn^{-/-} mice used in these experiments were bred and maintained at the Wadsworth Center Animal Core Facility under specific pathogen-free conditions. The majority of T cells in the DO11.10 and DO11.10 x Fyn^{-/-} mice are CD4⁺ cells, which bear a TCR that recognizes a chicken ovalbumin-derived

peptide, OVA_{323–339} (hereafter referred to as OVA), presented by I-A^d [26]. This TCR is encoded by transgenes encoding V β 8.2/V α 13.1 chains and can be identified by the anti-clonotypic mAb, KJ1-26 [27]. We have previously characterized the DO11.10 memory cells and their responses to peptide antigen and superantigen [28;29]. Fyn^{-/-} mice [30] were obtained from the Jackson Laboratory (Bar Harbor, ME) and backcrossed over 11 generations onto a BALB/c background, before they were bred to DO11.10 mice, to generate DO11.10 x Fyn^{-/-} mice. For all experiments, cells were obtained from mice that were 10–12 weeks old. Cells from both male or female mice were used, in different experiments, with no discernible differences in the results. All mice used in these studies were bred and maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (Washington, DC). All experiments were approved by the Wadsworth Center IACUC.

Reagents and antibodies

Mabs KJ1-26 (anti-DO11.10 clonotype) [27], C363.29B (anti-CD3e) [31], S4B6 (anti-IL-2) [32], XMG1.2 (anti-IFN- γ) [33], and 23G2 (anti-CD45RB) [34] were prepared from the supernatants of hybridoma cell lines, as previously described. Additional antibodies and probes used for immunoblot analysis and lymphokine analysis were anti-CD3 ζ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phosphotyrosine (G410) (Upstate Biotechnology, Lake Placid, NY), anti-phospho-ZAP-70 (Tyr319) (Cell Signaling Technology, Beverly, MA), and anti-IL-4, anti-CD3 ζ , anti-ZAP-70, goat anti-mouse IgG-HRPO and goat anti-rabbit IgG-HRPO (Transduction Laboratories, San Diego, CA). OVA peptide was synthesized and supplied by the Wadsworth Center Peptide Synthesis Core Facility. 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes, Inc., Eugene, OR), mitomycin C (Sigma Chemicals, St. Louis, MO), SU6656 (Calbiochem-Novabiochem Corp., San Diego, CA), and SEB (Toxin Technology, Sarasota, FL) were purchased.

Preparation of cells

In all experiments, enriched populations of CD4⁺ T cells were prepared by negative selection procedures as previously described [35], and were 90–95% CD4⁺ and <3% sIg⁺, as determined by flow-cytometric analyses. Naive and memory cells were separated as previously described [20] based upon CD45RB expression using mAb 23G2 supernatant and MACS (Miltenyi Biotec, Auburn, CA). There were no discernible differences between DO11.10 and DO11.10 x Fyn^{-/-} mice, in percentage of splenic cell populations, in number of total CD4⁺ cells, or in populations of naive and memory cells. OVA and SEB were presented to T cells using I-A^d/B7-1⁺ RT11-mB7 fibroblast cells [36], as previously described. Alternatively, APCs were prepared by T cell depletion of splenocytes using anti-Thy1-1.2 and complement, followed by anti-CD4 (mAb 2B6) and anti-CD8 plus complement [28]. Splenic APCs were treated with mitomycin C (25 μ g/ml) for 20 min at 37°C. For stimulation of T cells with anti-CD3, the cells were pre-coated with mAb C363.29B (rat anti-mouse CD3e) [31]; affipure F(ab')₂ fragment mouse-anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used to cross-link the mAb, to initiate activation.

Cell labeling and culture

For measurement of cell division using CFSE [37], DO11.10 and DO11.10 x Fyn^{-/-} CD4 cells were labeled with CFSE (5 μ M) prior to separation into naive and memory populations. DO11.10 naive and memory cells (1x10⁵/well) were cultured in 96-well flat-bottom plates with irradiated (10,000R) RT11-mB7 cells (2x10⁵/well) in 0.2 ml RPMI-1640 medium supplemented with 10% FBS, 50 μ M 2-mercaptoethanol, 100 U/ml penicillin, 100

μg/ml streptomycin, and 2 mM glutamine. Where indicated, SEB (20 μg/ml) or OVA_{323–339} (0.2 μg/ml) was added to the cultures. Where indicated, SU6656 (0.4 μM) was added to the cultures. Alternatively, we determined unlabeled T cell proliferation by the addition of 1 μCi/well of [³H]-thymidine for the final 16 hr of culture prior to harvesting the cells and determining cell-associated radioactivity. For experiments requiring two stimulation periods, irradiated RT11-mB7 cells were cultured for 3 hr at 37°C, to permit adherence. Non-adherent cells were removed, and the remaining cells were pulsed with SEB (20 μg/ml), or OVA_{323–339} (1.0 μg/ml) for 4 hr prior to addition of the CFSE-labeled T cells. For secondary stimulation, the T cells were removed, washed, and placed in 96-well U-bottom plates with T-cell depleted APCs (2x10⁵/well) and OVA_{323–339} (0.2 μg/ml). At the indicated times, the T cells were analyzed by flow cytometry, after staining with mAb KJ1-26, to identify the DO11.10 clonotype-bearing cells. Flow data was subsequently analyzed using FloJo software (Ashland, OR) to determine the amount of cell division in the cultures.

Immunoprecipitation and phosphoprotein detection—Irradiated RT11-mB7 (6x10⁶/well) cells were allowed to adhere to 24-well flat-bottom plates for 3 hr at 37°C. Non-adherent cells were removed, and the remaining cells were pulsed with SEB (20 μg/ml) or OVA_{323–339} (1.0 μg/ml) for 4 hr. T cells (3x10⁶/well) were added to the wells, and plates were centrifuged to facilitate cell-cell contact. Plates were then incubated at 37°C for various lengths of times. For anti-CD3 activation, T cells (3x10⁶ cells/50 μl) were incubated in RPMI with mAb C363.29B (20 μg/ml) [31] for 15 min on ice. After washing, the cells were resuspended in 50 μl RPMI with goat anti-rat F(ab')₂ (4 μg/3x10⁶ cells) and were incubated at 37°C. Regardless of the stimulus, signaling was quenched with ice-cold PBS, followed by centrifugation at 4°C. The cells were solubilized in cell lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 5 mM EDTA, 1% Triton X-100 (TX-100), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 mM iodoacetamide, 5 mM benzamidinium HCl, 1 mM AEBSF, 50 mM NaF, 10 mM Na₄P₂O₇, 1 mM Na₃VO₄) for 30 minutes at 4°C. Lysates were precleared with Bio Mag Protein-G magnetic microbeads (Qiagen Inc., Valencia, CA) for 20 min at 4°C, followed by removal of beads via adherence to magnets. Immunoprecipitations were carried out with 5 μg of primary antibody followed by magnetic separation using Protein-G magnetic microbeads. Proteins were separated using SDS-PAGE on 8 or 12% gels. Proteins were transferred to PVDF membranes, blocked with 3.5% non-fat dry milk dissolved in Tris-buffered saline Tween (TBST), and probed with specific protein antibodies and then by appropriate secondary antibodies (goat anti-mouse IgG-HRPO, goat anti-rabbit IgG-HRPO). Immunoblots were developed with the ECL+ system and autoradiographed on ECL Hyperfilm (Amersham Pharmacia Biotech, Piscataway, NJ). Where indicated, the immunoblots were re-probed [38] after removal of the bound antibodies by incubating the blots with a stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) at 50°C for 30 min. The developed immunoblots were analyzed by densitometry (UN-SCAN-IT; Silk Scientific, Orem, UT).

Lymphokine analysis—Percentages of lymphokine-producing cells were obtained via flow cytometry [39]. Briefly, CD4 cells were stimulated before a 5 hr stimulation with PMA (20 ng/ml) and ionomycin (1 μM) in the presence of monensin (2 μM). Cells were stained with mAb KJ1-26 to identify the antigen-specific T cells and were permeabilized with 0.1% saponin to allow staining and detection with of produced lymphokines with specific mAbs.

RESULTS

In BALB/c mice, unlike some other mouse strains (e.g.; C57BL/6), there is only a slight elevation of the surface molecule CD44 on “antigen-experienced” cells or memory cells, as compared to naive cells [7;40]. Separation of naive and memory cells on the basis of CD44

requires precise fluorescent cell sorting. Thus, to obtain sufficient cell numbers for our experiments, in our studies we used magnetic bead isolation of CD4 memory T cells based upon their lowered expression of the surface molecule CD45RB. CD45RB is highly expressed on murine naive CD4 cells and weakly expressed on memory cells [35]. However, similar results were obtained in independent experiments where the cells were flow sorted based on either high expression of CD44 or low expression of CD62L [7;41], instead of low expression of CD45B (WTL, unpublished studies). Using both non-transgenic BALB/c mice [13;14], and transgenic DO11.10 mice [20;21], we have previously described SEB-induced anergy in CD45RB^{lo} cells. Although CD45RB^{lo} cells can properly be called “antigen-experienced” (memory and effector) CD4 T cells, our starting cell populations were of small size and lacked the expression of activation markers, such as CD25 or CD69. Also, with respect to SEB-induced T cell anergy, given that activated cells, including effector cells and cloned cell lines, proliferate vigorously in response to SEB [9], we concluded that the anergic cells were initially resting memory cells. Although recent work has shown that multiple, distinct memory cell subsets exist (reviewed in [42]), our own prior work showed that anergy occurred in the entire population of splenic CD4 cells that were identified as being CD45RB^{lo} or CD44^{hi} or CD62L^{lo} [20;43] and thus, while using CD45B would most likely enrich for effector memory cells, in the following experiments we do not make distinctions between memory cell subsets. Regulatory T cells also possess low levels of CD45RB [44]. However, because in our studies the target T cells were CD25⁻, and because no proliferation or TCR proximal signaling was observed in the bulk CD45RB^{lo} cell population, we concluded that anergy did not occur through an indirect, suppressive mechanism. Further observation showed that no cytokines, including IL-10, were produced upon exposure of the cells to SEB [14]. However, direct indicators of regulatory T cell involvement, such as detection of Foxp3⁺ cells [45], were not evaluated.

In our experiments, we routinely used DO11.10 mice to obtain antigen-specific naive and memory CD4 T cells. We and others have shown that DO11.10 mice possess OVA-specific memory cells even if the mice had not previously been exposed to OVA [28;46]. The memory cells, generated in response to stimulation through a dual-expressed TCR composed of the transgenic TCR β chain paired with an endogenous TCR α chain, are indistinguishable from traditional memory cells in non-transgenic mice with respect to phenotype, biologic function, and signal transduction through the TCR. Our previous studies showed that TCR proximal signaling, initiated by peptide (OVA), was similar in DO11.10 naive and memory cells, and, in both cell types, signaling followed the canonical pathways described using conventional T cells [21].

Signal transduction in anergic memory cells is impaired

Previously, we showed that after 10–12 hr of exposure to SEB, CD4 memory T cells lost the ability to proliferate or secrete lymphokines when subsequently re-exposed to agonist peptide [20;21]. The *initial* signaling through the TCR, in response to SEB, was characterized by a failure of ZAP-70 to bind to phosphorylated CD3 ζ , as determined using a co-immunoprecipitation assay [21]. Consequently, ZAP-70 was not phosphorylated by the src kinase Lck and further signaling was truncated.

In the present study, we show that a similar defect in TCR proximal signaling exists in *anergic* memory cells when they are re-stimulated by agonistic OVA peptide. DO11.10 [26] CD45RB^{hi} (naive) and CD45RB^{lo} (memory) CD4⁺ T cells [28] were purified and cultured with APCs in the presence or absence of SEB. After 14 hr, the SEB was removed, and, the cells were re-stimulated with fresh APCs bearing OVA. For these experiments, the APCs, were RT11-mB7 cells [36]. Since these cells are adherent, analysis of the T cells was not hindered by contaminating APCs [20]. Following stimulation, the T cells were collected, and lysed, and ZAP-70 was then immunoprecipitated with a specific mAb. After SDS-

PAGE, ZAP-70 protein and (activated) p-ZAP-70 were identified using anti-ZAP-70 or anti-phosphotyrosine mAb, respectively. As we had previously observed, and as is shown in Figure 1a, direct stimulation of memory cells by OVA, or, of naive cells by either OVA or SEB, resulted in productive TCR-mediated signaling, as indicated by tyrosine phosphorylation of ZAP-70. In contrast, signaling in memory cells exposed to SEB was evidently impaired, given that there was a failure to activate ZAP-70. Further, in memory, but not naive, cells that had previously been exposed to SEB (*anergic*), ZAP-70 was not activated upon re-challenge with OVA. Hence, SEB not only blocks initial signaling, but also prevents subsequent signaling, even in response to a normally agonistic peptide.

In our previous study, we showed that activation and kinase activity of the src kinase Fyn was higher in memory cells that were exposed to SEB, as compared to either SEB-treated naive cells or in OVA-treated naive or memory cells [21]. To determine whether Fyn activity is also elevated in anergic cells, we cultured naive and memory cells with SEB for 14 hr, as above. The T cells were then recovered and stimulated for various lengths of time with OVA. Activation of Fyn was determined after specific immunoprecipitation and immunoblotting with anti-phosphotyrosine mAb. Although src kinases, including Fyn are phosphorylated at an inhibitory tyrosine residue in their inactive state, active kinase is associated with increased tyrosine phosphorylation. Initially, we observed little difference in pFyn levels in naive (nonanergic) or memory (anergic) cells cultured previously with or without SEB, suggesting that by 14 hours after the initial stimulation, Fyn signaling was greatly reduced and that exposure of memory cells to SEB did not lead to increased levels of constitutively activated Fyn (Figure 1b). Hence, the levels of pFyn were comparable in naive and memory cells, regardless of the prior stimulation with SEB. In this experiment, the immunoblots were over-exposed so as to better visualize the basal (presumably inactive) phosphorylation. Lesser exposure revealed only a faint or absent band, comparable to that shown in the lane of unstimulated naive cells in Figure 1c. Further, as indicated in Figure 1c, re-stimulation by OVA lead to higher levels of pFyn in anergic memory cells as compared to naive cells. Elevated Fyn activation cannot be due to a kinetic difference in signaling between the two cell types, since more pFyn is found in memory cells at all time points tested. Hence, decreased ZAP-70 activation and heightened Fyn activation occur not only in memory cells *initially* responding to SEB, but also in SEB-treated memory cells *subsequently* responding to OVA.

Inhibition of Fyn kinase permits memory T cells to proliferate in response to SEB

In order to determine whether the elevated Fyn kinase activity actively contributes to SEB-induced anergy in memory T cells, we examined memory cell responses, under conditions of reduced or absent Fyn activity. Initially, we used the chemical inhibitor SU6656 to block Fyn kinase. SU6656 reversibly inhibits both Lck and Fyn, but shows selectivity for Fyn at lower inhibitor concentrations [47]. In preliminary experiments, DO11.10 CD4 T cells were stimulated with OVA in the presence of increasing concentrations of SU6656 and then Fyn or Lck kinase activity was measured. Using an in vitro kinase assay, inhibition of Fyn, but not Lck, was observed at low concentrations of SU6656 ($>0.1 \mu\text{M}$), while, consistent with the findings of previous studies [47], both Lck and Fyn were inhibited only at the highest doses tested ($>5 \mu\text{M}$) (data not shown). We next determined the importance of Fyn kinase to memory cell proliferation and anergy, using a dose of SU6656 that inhibited only Fyn ($0.4 \mu\text{M}$). Proliferation was assessed in a CFSE-dilution assay [20;37], and the impact of SU6656 was determined. As previously shown [20], naive cells proliferated in response to either SEB (89% divided) or OVA (50%), as indicated by a reduction in intensity of CFSE fluorescence (Figure 2). In contrast, memory cells proliferated well in response to culture with OVA (47%) but not SEB (10%). Inhibition of Fyn activity apparently rescued the memory cells, given that the addition of SU6656 resulted in increased SEB-induced

proliferation (44%). We note that the addition of higher concentrations of SU6656 (>5 μ M) blocked both cell types from proliferating, in response to SEB or OVA (data not shown). This result is consistent with the inhibition of Lck, and blockade of normal TCR-mediated signaling.

To eliminate the possibility that SU6656 restores SEB-mediated proliferation by targeting a kinase other than Fyn, we next examined proliferative responses to SEB made by Fyn-deficient DO11.10 memory cells. We first characterized the splenic T cells from DO11.10 x Fyn^{-/-} mice and found that the cells were comparable to wild-type DO11.10 mice with respect to expression of the KJ1-26 (OVA-specific) clonotype [48] and also with respect to the frequency of CD45RB^{lo} memory cells (data not shown). Hence, as previously reported, the absence of Fyn does not impair CD4 T cell development or activation, nor does it prevent memory cell development [30]. We next determined whether memory cells from the DO11.10 x Fyn^{-/-} mice could respond to SEB or OVA. Previous studies have shown that Fyn is not required for normal antigen-driven T cell proliferation [30]; here we found, that both naive (not shown) and memory (70% divided) cells from Fyn-deficient mice proliferated well in response to stimulation with OVA. The absence of Fyn apparently altered the memory cell response to SEB, given that the Fyn⁻ cells (40%), in contrast to memory cells from wild-type DO11.10 mice (6%), proliferated when cultured in the presence of SEB (Figure 3a). Restoration was limited to SEB; neither wild-type nor Fyn⁻ memory cells responded well to soluble anti-CD3. Finally, to show that the requirement for Fyn in memory cell anergy was not unique to our transgenic model, we examined the responses of naive and memory CD4 T cells obtained from the original, B6/129 background, Fyn-deficient mice. Since, we could not stimulate the polyclonal T cells with specific peptide antigen, proliferation was induced by ConA. As we had previously observed using non-transgenic BALB/c mice, naive cells obtained from wild type B6/129 mice proliferated well in response to either SEB or soluble anti-CD3, whereas memory cells did not. In contrast, elimination of Fyn restored memory cell proliferation in response to SEB but not to soluble anti-CD3 (Figure 3b).

The inhibition of Fyn activity prevents SEB-mediated anergy development in memory T cells

The previous experiments indicated that either the absence of Fyn, or the inhibition of Fyn tyrosine kinase activity, enabled memory T cells to proliferate in response to culture with SEB. We next examined whether blocking Fyn during initial SEB signaling also allowed the cells to respond to re-stimulation by OVA. CD4⁺ T cells were cultured with APCs that presented SEB. Some of the cultures also contained SU6656, to inhibit Fyn, during this initial culture. SU6656 is a reversible inhibitor. By directly measuring Fyn kinase activity, we showed that, whereas Fyn was inhibited during the initial stimulation, activity could be re-induced after removal of SU6656 (data not shown). After 14 hr of culture with SEB (a time when initial proliferation was not yet stimulated) [20], the SEB and the inhibitor were withdrawn and the cells were re-stimulated by fresh APCs and OVA. As indicated in Figure 4a, inhibition of Fyn, during the initial exposure to SEB, prevented memory cell anergy, and thus permitted a subsequent proliferative response to OVA.

We also examined memory cell anergy in DO11.10 x Fyn^{-/-} mice. CFSE-labeled, naive and memory cells from wild-type or Fyn-deficient mice were cultured in the presence of SEB for 14 hr before removal of the SEB and then re-stimulation of the cells with OVA. As anticipated, memory cells from Fyn-deficient (48%), but not wild-type (9%), mice proliferated, confirming that the loss of Fyn rescued the cells from anergy (Figure 4b). Further, neither anergy nor the relief provided by the loss of Fyn were restricted to proliferative responses. When memory T cells from wild-type DO11.10 mice were cultured with SEB and subsequently re-stimulated with OVA, there was a marked reduction in

lymphokine production, as compared to the production in cells stimulated only with OVA (Table I). In contrast, exposure to SEB did not reduce lymphokine production by memory cells obtained from DO11.10 x Fyn^{-/-} mice. Taken together, these data suggest that SEB induces a Fyn-mediated signaling pathway that results in the loss of CD4 memory cell function.

Fyn-deficient memory cells exposed to SEB do not possess the signaling defects observed in Fyn-competent memory cells

As shown in our previous study on memory CD4 T cells initially exposed to SEB [21], and, also in the experiments in our current study that involved anergic memory cells responding to re-stimulation with peptide (Figure 1a), proximal signaling through the TCR is impaired. The initial block in signaling is characterized by a failure of binding between ZAP-70 and CD3 ζ . Consequently, ZAP-70 is not phosphorylated by Lck, and downstream signaling is abrogated. We wished to determine whether the rescue of cell proliferation and lymphokine secretion by Fyn inhibition can also restore productive TCR signaling. Accordingly, naive and memory CD4⁺ T cells were obtained from either wild-type DO11.10 mice or DO11.10 x Fyn^{-/-} mice, and the cells were stimulated, as above, with either OVA or SEB. As an additional control, soluble rat anti-mouse CD3 ϵ was used to stimulate the cells. As noted above, soluble anti-CD3 (cross-linked using F(ab')₂ mouse anti-rat IgG) stimulates naive, but not memory, cells to proliferate [15;49]. Unlike SEB, however, anti-CD3 does not cause memory cells to become anergic [20;49]. With respect to signaling in memory cells, anti-CD3 differs from SEB; the former does not promote heightened Fyn activation (data not shown) or proliferation in Fyn KO mice (Figure 3). Furthermore, analysis of TCR-mediated signaling showed that even though ZAP-70 does not become phosphorylated by Lck in memory cells exposed to anti-CD3, the coupling between ZAP-70 and CD3 ζ *does* occur [21]. Hence, when CD3 ζ was immunoprecipitated from lysates of naive and memory cells that had been exposed to OVA (or SEB- or anti-CD3-treated naive cells), pZAP-70 was co-precipitated (Figure 5a). Further, ZAP-70, but not pZAP-70, was found in CD3 ζ immunoprecipitates from memory cells exposed to anti-CD3. Neither pZAP-70 nor ZAP-70 protein was associated with memory cells exposed to SEB. However, when we examined lysates of SEB-treated memory cells from Fyn KO mice, we found that both ZAP-70 and pZAP-70 were associated with CD3 ζ (Figure 5b). In contrast, ZAP-70 associated with CD3 ζ , but was not phosphorylated, in anti-CD3-treated memory cells that lacked Fyn. Hence, the absence of Fyn led to restored cell proliferation and proximal signaling in memory cells that were exposed to SEB, but not in memory cells that were exposed to anti-CD3. cell function.

Fyn associates with c-Cbl in anergic memory cells

As indicated above, we observed an increased activity of Fyn kinase in anergic memory cells that were exposed to OVA after being cultured with SEB. Figure 1c shows the tyrosine phosphorylation of Fyn. However, when examining the entire blot, we found an additional phosphoprotein of higher molecular weight that had apparently co-immunoprecipitated with Fyn (Figure 6a). We identified this protein, which was more strongly phosphorylated in memory cells previously exposed to SEB, as c-Cbl (Figure 6b). This observation suggested that anergy is accompanied by a unique and stable association between Fyn and activated c-Cbl. To examine the role that the initial stimulation by SEB might play in c-Cbl activation, we stimulated naive and memory cells with SEB and at various times we examined cell extracts for the presence of phospho-c-Cbl and co-immunoprecipitated Fyn/c-Cbl complexes. We found that in memory cells exposed to SEB, c-Cbl phosphorylation appears to be greater and more sustained than in SEB-treated naive cells (or in memory cells stimulated by OVA (data not shown)) (Figure 6c). Note that phospho-c-cbl is undetectable by 14 hours after naive cell stimulation, while remaining present in memory cells. Further,

we observed an association between c-Cbl and Fyn at these later times (Figure 6c). Hence, by 7 hours, Fyn was found in c-Cbl immunoprecipitates from SEB-treated memory cells but not naive cells (Figure 6c) or in OVA-treated memory T cells (data not shown). Finally, we determined if Fyn activation was necessary for the increased levels of phospho-c-Cbl in memory cells exposed to SEB. Either DO11.10 or Fyn-deficient naive and memory cells were cultured with OVA or SEB and subsequently cell extracts were examined for the presence of phosphorylated c-Cbl. As above, c-Cbl was phosphorylated in response to cell stimulation with the highest level of activation found in SEB-treated memory cells (Figure 7). In contrast, when Fyn was absent, we found that little evidence for c-Cbl phosphorylation, regardless of the stimulus. These data suggest that Fyn is essential for normal c-Cbl activation in response to TCR signaling and that higher Fyn signal leads to a greater amount of activated c-Cbl.

DISCUSSION

Previous studies showed that although superantigens, such as SEB, bind to the TCR and promote signaling [21;23–25], they can elicit different signals than do peptide antigens. In resting memory CD4 cells, superantigen-mediated signaling leads to anergy and is associated with hyper-activation of Fyn kinase [21]. We now show that a second stimulation with peptide antigen induces higher levels of pFyn in those anergic cells, as compared to non-anergic cells. We also show that Fyn signaling is essential to SEB-induced anergy, given our finding that blockade of Fyn signaling enables cell activation. Finally, we provide evidence that Fyn, in association with c-cbl, disrupts canonical TCR signaling by interfering with the assembly of TCR-associated signaling complexes. ZAP-70 fails to become activated, because it is unable to bind to the CD3 ζ chain, and thus does not become accessible to Lck. Our current findings suggest that the reason for the lack of complex formation is that a Fyn-mediated signaling path is activated that prevents ZAP-70/CD3 interactions. In additional experiments, we show data to show that Fyn most likely acts by preventing the movement of ZAP-70 into the immunological synapse, where associations between ZAP-70 and the TCR complex can occur².

Anergy can be induced in memory CD4 T cells through exposure to low doses of specific peptide [17–19]. These studies found that TCR proximal signaling, including ZAP-70 activation, was impaired. As with SEB-induced signaling, Fyn activity was not decreased, however, increased Fyn activation was not reported. An interesting feature of peptide-induced anergy is the primary role that B cells play as the anergy-inducing APC. In our own studies, T-depleted splenocytes, primarily B cells, were used to present SEB and induce memory cell anergy. However, we subsequently observed showed that an artificial APC (e.g.; RT11-mB7 cells [36]) could effectively promote anergy. Further, we also found that the inclusion of CTLA-4-specific blocking antibodies did not relieve anergy as they did for peptide-induced anergy [50]. Hence, we suggest that superantigens, low avidity peptide interactions, and soluble anti-CD3 all reveal distinct and unique pathways for specifically regulating memory cell activation.

The involvement of src kinase signaling in T cell responses to superantigens is complex, and there is evidence that, even within naive cell populations, unique responses are made to superantigens. For example, studies, largely focusing on Lck, have suggested that canonical TCR proximal signaling is not required for responses to superantigens [23;51]. Hence, Lck-deficient Jurkat (JCAM-1) cells produced IL-2 in response to the superantigen SEE. Likewise, inhibition of src kinases with PP2 blocked anti-CD3, but not SEE-induced

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stimulation of peripheral blood mononuclear cells (PBMC) [23]. Additional studies suggested that Jurkat cells or PBMCs could also respond to SEE, even when ZAP-70 was not activated [24]. Our observations on memory cells differ from these studies, in that SEB did not stimulate proliferation in the absence of ZAP-70 activation.

When memory CD4 cells were initially stimulated by SEB, or when these cells were later re-stimulated by OVA, no proliferation or cytokine secretion was observed. Further, proximal signaling, as evidenced by a failure of ZAP-70 to associate with CD3 ζ , was impaired. Hence, unlike the situation in some forms of peptide- or adjuvant-induced anergy [52], initial cell proliferation was not required here. We also conclude that the same mechanistic signals, that prevent initial proliferation, are also the signals that operate so as to prevent subsequent activation in response to agonist peptide. This commonality suggests that alternative signaling paths are quickly activated and that negative signaling is maintained as long as the cells are anergic. Our data suggest that Fyn kinase is a prominent player in that pathway. Fyn is quickly activated upon exposure to SEB, its activation is sustained in anergic cells, and the inhibition or absence of Fyn prevents anergy. Fyn has been implicated in some tolerance models, as indicated by increased expression or activity in anergic cells [53–57]. Elimination of Fyn expression in a population of anergic double negative T cells led to upregulation of CD25, increased sensitivity to IL-2, and, consequently, increased proliferation [57]. This contrasts with our observation that memory cell anergy is not due to a failure to upregulate CD25 or respond to IL-2 [14]. Thus, Fyn plays multiple, distinct roles in anergy induction. The mechanism by which increased Fyn activity could promote SEB-induced anergy in memory T cells is, at present, unclear. One potential means could be through activation of the E3 ubiquitin ligase, c-Cbl. Previous studies showed that C-Cbl is inducibly phosphorylated by Fyn [58–60] and, likewise, we find c-Cbl to remain non-phosphorylated in Fyn-deficient memory cells. C-Cbl can modulate ZAP-70 signaling in thymocytes and T cells through various mechanisms, such as recruitment of phosphatases to deactivate ZAP-70, ubiquitination and down-regulation of ZAP-70, or, blockage of recruitment of ZAP-70 to the CD3 complex [61–63]. Likewise, we observed an increase in c-Cbl activation and increased Fyn/c-Cbl complex formation in anergic memory cells. We speculate that Fyn regulates the localization of ZAP-70 through c-Cbl. Our ongoing studies will address whether c-Cbl directly contributes to SEB-induced anergy and, if so, how c-Cbl prevents ZAP-70 activation.

Unlike naive cells, memory cells are directly induced to become anergic upon exposure to SEB. We speculate that superantigens exploit a self-tolerance mechanism unique to memory cells, although the normal trigger of this pathway is, at present, unclear. We have also previously suggested that, because superantigens may be encountered normally during infection, memory cell anergy may shape the immune repertoire [20]. Tolerance of entire families of memory cells might eliminate the response to immunodominant antigens on the bacterial surface and facilitate pathogen evasion. Further, since most of the anergic T cells would not be (superantigen-producing) pathogen-specific, exposure to a single or multiple superantigens may have implications on the protection imparted by prior vaccination to multiple pathogens. Indeed, in additional studies we found that inoculation of live animals with SEB prevented recall CD4-dependent immune responses to unrelated antigens after vaccination (manuscript in preparation). Hence, understanding the mechanism underlying this form of tolerance may have implications to both autoimmune and protective responses.

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References

1. Vitetta ES, Berton MT, Burger C, Kepron M, Lee WT, Yin XM. Memory T and B cells. *Annu Rev Immunol.* 1991; 9:193–217. [PubMed: 1910676]
2. Dutton RW, Bradley LM, Swain SL. T cell memory. *Annu Rev Immunol.* 1998; 16:201–223. [PubMed: 9597129]
3. Swain SL. Helper T cell differentiation. *Curr Opin Immunol.* 1999; 11:180–185. [PubMed: 10322154]
4. Croft M, Dubey C. Accessory molecule and costimulation requirements for CD4 T cell response. *Critical Reviews in Immunology.* 1997; 17:89–118. [PubMed: 9034725]
5. Hogquist KA, Baldwin TA, Jameson SC. Central tolerance: learning self-control in the thymus. *Nat Rev Immunol.* 2005; 5:772–782. [PubMed: 16200080]
6. Sanders ME, Makgoba MW, Sharrow SO, Stephany D, Springer TA, Young HA, Shaw S. Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL-1, CDw29, and Pgp-1) and have enhanced IFN-gamma production. *J Immunol.* 1988; 140:1401–1407. [PubMed: 2894392]
7. Lee WT, Vitetta ES. The differential expression of homing and adhesion molecules on virgin and memory T cells in the mouse. *Cell Immunol.* 1991; 132:215–222. [PubMed: 1676613]
8. Ernst DN, Hobbs MV, Torbett BE, Glasebrook AL, Rehse MA, Bottomly K, Hayakawa K, Hardy RR, Weigle WO. Differences in the expression profiles of CD45RB, Pgp-1 and 3G11 surface antigens in the patterns of IL-2 and IL-4 secretion by splenic CD4⁺ T cells from young and aged mice. *FASEB J.* 1990; 4:2042.
9. Janeway CA Jr, Yagi J, Conrad PJ, Katz ME, Jones B, Vroegop S, Buxser S. T-cell responses to M1s and to bacterial proteins that mimic its behavior. *Immunol Rev.* 1989; 107:61–88. [PubMed: 2522086]
10. Herman A, Kappler JW, Marrack P, Pullen AM. Superantigens: mechanism of T-cell stimulation and role in immune responses. *Annu Rev Immunol.* 1991; 9:745–772. [PubMed: 1832875]
11. Fraser J, Arcus V, Kong P, Baker E, Proft T. Superantigens - powerful modifiers of the immune system. *Mol Med Today.* 2000; 6:125–132. [PubMed: 10689316]
12. Marrack P, Kappler J. The staphylococcal enterotoxins and their relatives. *Science.* 1990; 248:705–711. [PubMed: 2185544]
13. Lee WT, Vitetta ES. Memory T cells are anergic to the superantigen, staphylococcal enterotoxin B. *J Exp Med.* 1992; 176:575–580. [PubMed: 1500861]
14. Lee WT, Thrush GR, Vitetta ES. Staphylococcal enterotoxin B induces the expression of activation markers on murine memory T cells in the absence of proliferation or lymphokine secretion. *Cell Immunol.* 1995; 162:26–32. [PubMed: 7704907]
15. Luqman M, Bottomly K. Activation requirements for CD4⁺ t-cells differing in CD45r expression. *J Immunol.* 1992; 149:2300–2306. [PubMed: 1388189]
16. Farber DL, Luqman M, Acuto O, Bottomly K. Control of memory CD4 T cell activation: MHC class II molecules on APCs and CD4 ligation inhibit memory but not naive CD4 cells. *Immunity.* 1995; 2:249–259. [PubMed: 7535181]
17. Dalai SK, Mirshahidi S, Morrot A, Zavala F, Sadegh-Nasseri S. Anergy in memory CD4⁺ T cells is induced by B cells. *J Immunol.* 2008; 181:3221–3231. [PubMed: 18713993]
18. Mirshahidi S, Ferris LC, Sadegh-Nasseri S. The magnitude of TCR engagement is a critical predictor of T cell anergy or activation. *J Immunol.* 2004; 172:5346–5355. [PubMed: 15100274]
19. Mirshahidi S, Huang CT, Sadegh-Nasseri S. Anergy in peripheral memory CD4(+) T cells induced by low avidity engagement of T cell receptor. *J Exp Med.* 2001; 194:719–731. [PubMed: 11560989]
20. Watson AR, Mittler JN, Lee WT. Staphylococcal enterotoxin B induces anergy to conventional peptide in memory T cells. *Cell Immunol.* 2003; 222:144–155. [PubMed: 12826084]

21. Watson AR, Lee WT. Defective T cell receptor-mediated signal transduction in memory CD4 T lymphocytes exposed to superantigen or anti-T cell receptor antibodies. *Cell Immunol.* 2006; 242:80–90. [PubMed: 17083922]
22. Zamoyska R. Superantigens: supersignalers? *Sci STKE.* 2006:e45.
23. Bueno C, Criado G, McCormick JK, Madrenas J. T cell signalling induced by bacterial superantigens. *Chem Immunol Allergy.* 2007; 93:161–180. [PubMed: 17369705]
24. Bueno C, Lemke CD, Criado G, Baroja ML, Ferguson SS, Rahman AK, Tsoukas CD, McCormick JK, Madrenas J. Bacterial superantigens bypass Lck-dependent T cell receptor signaling by activating a $\alpha 11$ -dependent, PLC- β -mediated pathway. *Immunity.* 2006; 25:67–78. [PubMed: 16860758]
25. Criado G, Madrenas J. Superantigen stimulation reveals the contribution of Lck to negative regulation of T cell activation. *J Immunol.* 2004; 172:222–230. [PubMed: 14688329]
26. Murphy KM, Heimberger AB, Loh DY. Induction by antigen of intrathymic apoptosis of CD4⁺CD8⁺TCR^{lo} thymocytes in vivo. *Science.* 1990; 250:1720–1722. [PubMed: 2125367]
27. Haskins K, Kubo R, White J, Pigeon M, Kappler J, Marrack P. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. *J Exp Med.* 1983; 157:1149–1169. [PubMed: 6601175]
28. Lee WT, Cole-Calkins J, Street NE. Memory T cell development in the absence of specific antigen priming. *J Immunol.* 1996; 157:5300–5307. [PubMed: 8955176]
29. Lee WT, Shiledar-Baxi V, Winslow GM, Mix D, Murphy DB. Self-restricted dual receptor memory T cells. *J Immunol.* 1998; 161:4513–4519. [PubMed: 9794376]
30. Stein PL, Lee HM, Rich S, Soriano P. 59^{fyn} mutant mice display differential signaling in thymocytes and peripheral T cells. *Cell.* 1992; 70:741–750. [PubMed: 1387588]
31. Portoles P, Rojo J, Golby A, Bonneville M, Gromkowski S, Greenbaum G, Janeway CA Jr, Murphy DB, Bottomly K. Monoclonal antibodies to murine CD3 ϵ define distinct epitopes, one of which may interact with CD4 during T cell activation. *J Immunol.* 1989; 142:4169–4175. [PubMed: 2470817]
32. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol.* 1986; 136:2348–2357. [PubMed: 2419430]
33. Cherwinski HM, Schumacher JH, Brown KD, Mosmann TR. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between T_{H1} and T_{H2} clones revealed by RNA hybridization, functionally monospecific bioassays and monoclonal antibodies. *J Exp Med.* 1987; 166:1229–1233. [PubMed: 2960769]
34. Birkeland ML, Metlay J, Sanders VM, Fernandez-Botran R, Vitetta ES, Steinman RM, Pure E. Epitopes on CD45R (T200) molecules define differentiation antigens on murine B and T lymphocytes. *J Mol Cell Immunol.* 1988; 4:71–85. [PubMed: 2475124]
35. Lee WT, Yin X-M, Vitetta ES. Functional and ontogenetic analysis of murine CD45R_{hi} and CD45R_{lo} CD4⁺ T cells. *J Immunol.* 1990; 144:3288–3295. [PubMed: 1970350]
36. Zhou T, Weaver C, Linsley PS, Mountz JD. T cells of staphylococcal enterotoxin B-tolerized autoimmune MRL-*lpr/lpr* mice require co-stimulation through the B7- CD28/CTLA-4 pathway for activation and can be reenergized *in vivo* by stimulation of the T cell receptor in the absence of this co-stimulatory signal. *Eur J Immunol.* 1994; 24:1019–1025. [PubMed: 7514125]
37. Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. *J Immunol Methods.* 1994; 171:131–137. [PubMed: 8176234]
38. Kaufmann SH, Ewing CM, Shaper JH. The erasable Western blot. *Anal Biochem.* 1987; 161:89–95. [PubMed: 3578791]
39. Prussin C, Metcalfe DD. Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies. *J Immunol Methods.* 1995; 188:117–128. [PubMed: 8551029]
40. Lynch F, Ceredig R. Mouse strain variation in Ly-24 (PgP-1) expression by peripheral T cells and thymocytes: implications for T cell differentiation. *Eur J Immunol.* 1989; 19:223–220. [PubMed: 2784763]

41. Ernst DN, Hobbs MV, Torbett BE, Glasebrook AL, Rehse MA, Bottomly K, Hayakawa K, Hardy RR, Weigle WO. Differences in the expression profiles of CD45RB, Pgp-1, and 3G11 membrane antigens and in the patterns of lymphokine secretion by splenic CD4⁺ T cells from young and aged mice. *J Immunol.* Sep; 1990 145(5):1295–1302. [PubMed: 1974562]
42. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol.* 2004; 22:745–763. [PubMed: 15032595]
43. Lee WT, Pelletier WJ. Visualizing memory phenotype development after *in vitro* stimulation of CD4⁺ T cells. *Cell Immunol.* 1998; 188:1–11. [PubMed: 9743552]
44. Sakaguchi S. Naturally arising CD4⁺ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol.* 2004; 22:531–562. [PubMed: 15032588]
45. Sakaguchi S. Naturally arising Foxp3-expressing CD25⁺CD4⁺ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol.* 2005; 6:345–352. [PubMed: 15785760]
46. Saparov A, Kraus LA, Cong Y, Marwill J, Xu XY, Elson CO, Weaver CT. Memory/effector T cells in TCR transgenic mice develop via recognition of enteric antigens by a second, endogenous TCR. *Int Immunol.* 1999; 11:1253–1264. [PubMed: 10421783]
47. Blake RA, Broome MA, Liu X, Wu J, Gishizky M, Sun L, Courtneidge SA. SU6656, a Selective Src Family Kinase Inhibitor, Used To Probe Growth Factor Signaling. *Molecular and Cellular Biology.* 2000; 20:9018–9027. [PubMed: 11074000]
48. Kappler J, Kubo R, Haskins K, Hannum C, Marrack P, Pigeon M, McIntyre B, Allison J, Trowbridge I. The major histocompatibility complex-restricted antigen receptor on T cells in mouse and man: identification of constant and variable peptides. *Cell.* 1983; 35:295–302. [PubMed: 6605199]
49. Farber DL, Acuto O, Bottomly K. Differential T cell receptor-mediated signaling in naive and memory CD4 T cells. *Eur J Immunol.* 1997; 27:2094–2101. [PubMed: 9295050]
50. Lee WT, Mittler JN, Watson ARO, Li X. Activation and anergy in memory CD4 T lymphocytes. *Rec Res Dev Immunol.* 2002; 4:303–329.
51. Criado G, Madrenas J. Superantigen stimulation reveals the contribution of Lck to negative regulation of T cell activation. *J Immunol.* 2004; 172:222–230. [PubMed: 14688329]
52. Kearney ER, Pape KA, Loh DY, Jenkins MK. Visualization of peptide-specific immunity and peripheral tolerance induction in vivo. *Immunity.* 1994; 1:327–339. [PubMed: 7889419]
53. Boussiotis VA, Freeman GJ, Berezovskaya A, Barber DL, Nadler LM. Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1. *Science.* 1997; 278:124–128. [PubMed: 9311917]
54. Gajewski TF, Qian D, Fields P, Fitch FW. Anergic T-lymphocyte clones have altered inositol phosphate, calcium, and tyrosine kinase signaling pathways. *Proc Natl Acad Sci USA.* 1994; 91:38–42. [PubMed: 7506419]
55. Gajewski TF, Fields P, Fitch FW. Induction of the increased Fyn kinase activity in anergic T helper type 1 clones requires calcium and protein synthesis and is sensitive to cyclosporin A. *Eur J Immunol.* 1995; 25:1836–1842. [PubMed: 7621861]
56. Utting O, Teh SJ, Teh HS. A population of in vivo anergized T cells with a lower activation threshold for the induction of CD25 exhibit differential requirements in mobilization of intracellular calcium and mitogen-activated protein kinase activation. *J Immunol.* 2000; 164:2881–2889. [PubMed: 10706673]
57. Utting O, Priatel JJ, Teh SJ, Teh HS. p59fyn (Fyn) promotes the survival of anergic CD4-CD8- $\alpha\beta$ TCR⁺ cells but negatively regulates their proliferative response to antigen stimulation. *J Immunol.* 2001; 166:1540–1546. [PubMed: 11160194]
58. Deckert M, Elly C, Altman A, Liu YC. Coordinated regulation of the tyrosine phosphorylation of Cbl by Fyn and Syk tyrosine kinases. *J Biol Chem.* 1998; 273:8867–8874. [PubMed: 9535867]
59. Hunter S, Burton EA, Wu SC, Anderson SM. Fyn associates with Cbl and phosphorylates tyrosine 731 in Cbl, a binding site for phosphatidylinositol 3-kinase. *J Biol Chem.* 1999; 274:2097–2106. [PubMed: 9890970]

60. Tezuka T, Umemori H, Fusaki N, Yagi T, Takata M, Kurosaki T, Yamamoto T. Physical and functional association of the cbl protooncogen product with an src-family protein tyrosine kinase, p53/56lyn, in the B cell antigen receptor-mediated signaling. *J Exp Med.* 1996; 183:675–680. [PubMed: 8627181]
61. Murphy MA, Schnall RG, Venter DJ, Barnett L, Bertoncello I, Thien CB, Langdon WY, Bowtell DD. Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice. *Mol Cell Biol.* 1998; 18:4872–4882. [PubMed: 9671496]
62. Luper ML Jr, Rao N, Eck MJ, Band H. The Cbl protooncoprotein: a negative regulator of immune receptor signal transduction. *Immunol Today.* 1999; 20:375–382. [PubMed: 10431158]
63. Thien CB, Bowtell DD, Langdon WY. Perturbed regulation of ZAP-70 and sustained tyrosine phosphorylation of LAT and SLP-76 in c-Cbl-deficient thymocytes. *J Immunol.* 1999; 162:7133–7139. [PubMed: 10358158]

HIGHLIGHTS

- A bacterial toxin (SEB) causes memory CD4 T cell inactivation (anergy)
- We show that anergy is dependent upon activation of Fyn kinase
- Inhibition or loss of Fyn kinase prevents anergy
- We show that Fyn associates with c-Cbl in response to SEB
- Fyn/c-Cbl complexes prevents ZAP-70 from binding to the TCR/CD3 complex

Figure 1. ZAP-70 is not activated, but fyn is hyper-activated, in anergic memory CD4 cells
a. Memory (*M*) and naive (*N*) DO11.10 T cells were stimulated for 2 min with SEB or with OVA, or they were left unstimulated (*Unstim.*). Some cells, initially stimulated with SEB for 14 hrs, were then re-stimulated with OVA (*SEB + OVA*). ZAP-70 was immunoprecipitated (*ip*). Immunoblotting (*blot*) was performed, as indicated, with anti-phosphotyrosine and ZAP-70 Abs. *b.* Memory and naive DO11.10 T cells were stimulated with SEB for 14 hr or left unstimulated before cell lysis and analysis of pfyn, or *c.* alternatively, the SEB-stimulated cells were re-stimulated for the indicated times by OVA. Unstimulated cells are not shown in Figure 1c, but they are the same as in naive cells (Figure 1b). Fyn protein was immunoprecipitated, and immunoblotting was performed, as indicated, with anti-phosphotyrosine and anti-fyn Abs. Densitometry was performed on the immunoblots and the

relative levels of phosphoprotein are shown (*b*) or expressed as a ratio relative to the total amount of precipitated protein (*a,c*). Data are representative of two independent experiments.

Figure 2. Inhibition of Fyn permits memory cells to proliferate in response to SEB

Naive and memory DO11.10 CD4 T cells were labeled with CFSE and then stimulated in vitro with OVA or SEB in the presence or absence of SU6656. After 3.5 days, proliferation was assessed by flow cytometry, measuring CFSE fluorescence on KJ1-26⁺ cells. In the histograms the number in each panel represents the percentage of cells with decreased fluorescence as compared to the undivided peak. Data are representative of three independent experiments.

Figure 3. Fyn-deficient memory cells proliferate in response to SEB

a. Memory CD4 T cells from DO11.10 and DO11.10 x Fyn^{-/-} mice were labeled with CFSE and then stimulated in vitro with soluble anti-CD3, SEB, or OVA. After 3.5 days, proliferation was assessed by flow cytometry. In the histograms The number in each panel represents the percentage of cells with decreased fluorescence as compared to the undivided peak. Data are representative of three independent experiments. *b.* Memory (*gray bars*) or Naive (*white bars*) cells from the original Fyn^{-/-} strain (*hatched*) or their wild-type littermates (*open*) were cultured with soluble anti-CD3, SEB, or ConA. After 3 days proliferation was determined by [³H]-thymidine incorporation.

Figure 4. Suppression of Fyn prevents SEB-induced memory T cell anergy and enables cell proliferation in response to agonist peptide

DO11.10 naive and memory CD4 T cells were labeled with CFSE and then stimulated for 14 hr with SEB, washed, and re-stimulated with OVA. After an additional 3.5 days, proliferation was assessed by flow cytometry. *a*. Cells were cultured with SEB and in the presence or absence of SU6656. *b*. Cultures contained cells from either wild-type (*WT*) or *Fyn*-deficient DO11.10 mice. In the histograms, the number in each panel represents the percentage of cells with decreased fluorescence as compared to the undivided peak. Data are representative of two independent experiments.

Figure 5. Suppression of Fyn restores TCR proximal signaling in response to SEB

Naive and memory T cells from (a) DO11.10, or, (b). DO11.10 x *Fyn*^{-/-} mice were stimulated for 2 min with OVA, SEB, or anti-CD3, or were left unstimulated (*Unstim.*), and CD3 ζ -chain was then immunoprecipitated. Immunoblotting was performed, as indicated, using anti-phosphotyrosine, anti-CD3 ζ , anti-ZAP-70, or anti-pY319 ZAP-70 (activated ZAP-70). Data are representative of three independent experiments.

Figure 6. C-Cbl association with Fyn and activation is increased in memory cells exposed to SEB
a, b. As in Figure 1c, memory and naive DO11.10 T cells were stimulated with SEB for 14 hr, washed, and then re-stimulated for the indicated times by OVA. Fyn protein was immunoprecipitated, and immunoblotting was performed, as indicated, with *a.* anti-phosphotyrosine and *b.* anti-c-Cbl Abs. *c.* Freshly isolated memory and naive DO11.10 T cells were stimulated by SEB for the indicated times and c-Cbl was then immunoprecipitated. Immunoblotting was performed, as indicated, using anti-phosphotyrosine, anti-c-Cbl, and anti-Fyn. Data are representative of two independent experiments.

Figure 7. The increase in C-Cbl activation in SEB-treated memory cells is dependent on Fyn
 Naive and memory T cells from DO11.10 or DO11.10 x Fyn^{-/-} mice were stimulated for 10 min with OVA or SEB, or were left unstimulated (*Unstim*), and c-Cbl was then immunoprecipitated. Immunoblotting was performed, as indicated, using anti-phosphotyrosine or anti-c-Cbl. Data are representative of two independent experiments.

Table 1

Lymphokine production following pre-exposure to SEB^a

	WT		Fyn ^{-/-}	
	Pre-culture ^b	-	SEB	-
OVA-induced lymphokine ^c	IL-2	33.0	10.1	30.0
	IL-4	4.7	0.9	2.1
	IFN-γ	10.4	0.9	12.3

^a Lymphokine production was measured in memory CD4 T cells from either DO11.10 (WT) or DO11.10 x Fyn^{-/-} (Fyn^{-/-}) mice. The data depicted are representative of two separate experiments.

^b Memory T cells were stimulated by adherent APCs (RT11-mB7) pulsed with nothing or SEB (20 μg/ml). After 14 hours, the T cells were removed, washed, and then re-stimulated by APCs (T-depleted splenocytes) presenting OVA (0.1 μg/ml).

^c percentage of KJ1-26⁺ cells containing IL-2, IL-4, or IFN-γ, as determined by intracellular staining after 60 h of culture with OVA